THE MUTABILITY OF BACILLUS ANTHRACIS SPORES DURING GERMINATION[1]

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The behavior of bacteria subjected to the influence of mutating agents has been studied in considerable detail, but the interpretation of the results is still largely dependent on analogy to those obtained with higher forms. The lack of clear-cut cytological evidence of a nuclear cycle has been a handicap to the bacteriologist. Studies with *Tradescantia* microspores by Sax (1940) showed considerable variation in the ease with which chromosomal aberrations could be induced during the various phases of the nuclear cycle. Aging of *Drosophila* sperm and of seed (cf. Sax) increased the yield of mutations induced by radiations. Clark *et al.* (1950) and Haas *et al.* (1948) have studied the effect of mutating agents on *Escherichia coli* strains preceding sexual recombination, but the analysis was limited to the few pertinent biochemical deficiencies; no analysis was made of the effect of mutagens, applied during the course of association of the recombining strains, on other genetically controlled traits. Since it is possible that only a small fraction of the organisms in such mixtures of two recombining strains is actually involved in the transfer of genetic material, a change in mutability during the process might be difficult to evaluate.

A definite change in the disposition of nuclear material has been reported in the transition of bacterial vegetative cells to spores (Kleineberger-Nobel, 1947; Delaporte, 1950). A process akin to reduction division appears to occur, although the actual observation of chromosome numbers cannot be made. During spore germination the nuclear material returns to the state encountered in the vegetative cell. The effect of mutagenic agents on bacteria during such transition stages has not been reported previously and should yield additional information on the mechanism controlling heredity in these microorganisms. Although we have studied the quantitative aspects of ultraviolet-induced mutations through the entire spore to spore cycle of *Bacillus anthracis*, this report emphasizes the results during the germination process. Spore formation with this organism in nutrient broth extends over a considerable period of time, and therefore a quantitative estimation of the effects of mutagenic action on the developing spore is difficult to obtain; spore germination, however, can be controlled more readily. As a measure of mutability we employed the convenient and widely used mutation to streptomycin resistance.

Few reports on the induction of mutations in bacterial spores have come to

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our attention. Burkholder and Giles (1947) and Guthrie and Saperstein (1949) obtained biochemical mutants by irradiating *Bacillus subtilis* spores with ultraviolet light. A mutant of *Bacillus globigii* that yielded heat-resistant spores was obtained by Davis and Williams (1948), but this was obtained by repeated selection of survivors from thermal killing experiments.

In some respects the mutation of spores presents situations differing from those encountered in vegetative cells. Cytological evidence suggests that the spore may be the haploid phase in the life cycle of a sporogenous bacterium. Burkholder and Giles felt that the high incidence of "zero-point" mutants in irradiated spores supported this view. In the vegetative cell the nuclear material is surrounded by cytoplasm that offers protection to the gene material and increases the likelihood of indirect action of radiations; in the spore the cytoplasm is greatly reduced and the nuclear material is concentrated in a small area outside the cytoplasm and directly beneath the exine. The spore is tremendously more resistant than the vegetative cell to the killing action of chemical agents and heat, and somewhat more resistant (though the difference is not nearly so great) to the killing action of radiations. Although subject to considerable speculation, the reasons for these differences are unknown. Spores from the same culture may vary widely in morphology, heat resistance, refringence, and associated characteristics (Knaysi, Baker, and Hillier, 1947). That these differences need not be genotypic may be observed from a study of the progeny of those aberrant in morphology and refringence. A population of spores of one genotype presenting numerous phenotypes is to be expected since it is not possible to control the environment for that extended period when the spores are formed in the development of a culture. This phenotypic variation in a population of spores of one genotype is a problem to be considered when these forms are employed. The recent trend in attributing to a genetic origin all differences in microorganisms is a result of the use of rigidly controlled conditions and active log phase cultures in which—except for mutants—little variation exists. On the other hand, genotypic variation to antibiotic resistance in a population of spores may be obscured by a greater sensitivity of the germination process than of the vegetative growth; a spore may fail to germinate in a concentration of antibiotic to which its vegetative progeny are resistant mutants.

EXPERIMENTAL METHODS

The Vollum strain 994 of *Bacillus anthracis*, obtained from Camp Detrick, Maryland, was used in all experiments. Spores were prepared by growing organisms upon nutrient agar flats and harvesting at 5 days when most of the cell walls had lysed leaving spores free from shielding material. The suspension was washed, filtered through cotton, and suspended in M/200 phosphate buffer at pH 7.0. All such preliminary manipulations were carried out with refrigerated materials. Routine microscopic and cultural examinations were made, but pathogenicity was not checked. The nutrient broth or agar (Difco) employed for culture and plating contained 0.1 per cent glucose, and 0.1 per cent peptone water was used for dilution. Dilutions were plated immediately, and final readings of plate counts

were made after 3 days. All broth cultures were incubated on a reciprocating shaker at 37 C. Streptomycin sulfate furnished by the Abbott Laboratories, Chicago, Illinois, was prepared as a concentrated stock solution 24 hours prior to use to allow self-sterilization before incorporation into agar for assaying the numbers of resistant mutants.

Twenty-five ml of the cell suspension were placed in a 1-liter, cold, sterile beaker containing a small glass-incased nail for use with a magnetic stirrer. The Hanovia double-U SC 2537 quartz ultraviolet lamp, operating at 120 milliamperes, was preheated prior to use. The surface of the suspension was 21.6 cm from the center of the lamp, and was agitated at a constant rate by the magnetic stirrer. At measured time intervals 1.5-ml aliquots were withdrawn and placed in cold, sterile test tubes from which the total survivors and the zero point mutants were determined, and an inoculation into a nutrient medium was made to permit the estimation of end point mutants after development of the culture (Demerec and Latarjet, 1947).

EXPERIMENTAL RESULTS

Preliminary to the mutability studies it was necessary to obtain data on the incidence of spontaneous mutations in the population and on the lethal effect of ultraviolet during the germination period. When washed spores of *B. anthracis* were inoculated into nutrient broth at a rate of 10.9 million per ml and plated at measured intervals on streptomycin agar, the incidence of spontaneous mutants in the population remained relatively constant. This is tabulated in table 1. Not only during this transition phase of spores to vegetative cells but in measurements made throughout the growth period we found no significant changes in the equilibrium of the streptomycin-resistant mutants and the rest of the population.

The killing curves on the germinating spores were obtained by inoculating nutrient broth at a rate of 200 million spores per ml, holding it at 37 C for the indicated time, cooling it rapidly by icing, and irradiating the cold broth spore suspension. Figure 1 shows the family of log survival curves that resulted. When the spores were irradiated without a germination period, it required 720 seconds to obtain 99.99 per cent killing; after a germination period of only 5 minutes the time required for an equivalent lethality was reduced to about 640 seconds. Further incubation increased the sensitivity until after 80 minutes it approached that of the vegetative culture. The numbers of spores undergoing change during these periods can be appreciated better by observing some point on the survival curve, such as after 240 seconds of irradiation, where 56 million ungerminated spores survived but only 25 million of those germinated for 5 minutes, 16 million of those germinated for 10 minutes, and 1 million of those germinated for 80 minutes. The term "germinated" as used here implies only that the spores were placed under conditions favorable to this event, because even after 80 minutes the majority of the spores showed no visible change in cytology or staining characteristics. With incubation periods longer than 80 minutes the results were erratic because of the fact that some cell division occurred. It is

evident that, long before the appearance of any cytological evidence of germination, there are changes in the spore that render it sensitive to ultraviolet light.

TABLE 1

Incidence of streptomycin-resistant mutants during germination of B. anthracis

GERMINATION TIME	MUTANTS/MILLION (μG/ML STREPTOMYCIN)			
	0.75	1.0	2.0	3.0
0	530	320	90	0
5	900	270	100	0
10	520	280	100	0
15	810	270	100	0
20	840	270	90	0
40	570	200	100	0
80	530	210	110	0
160	520	200	100	0

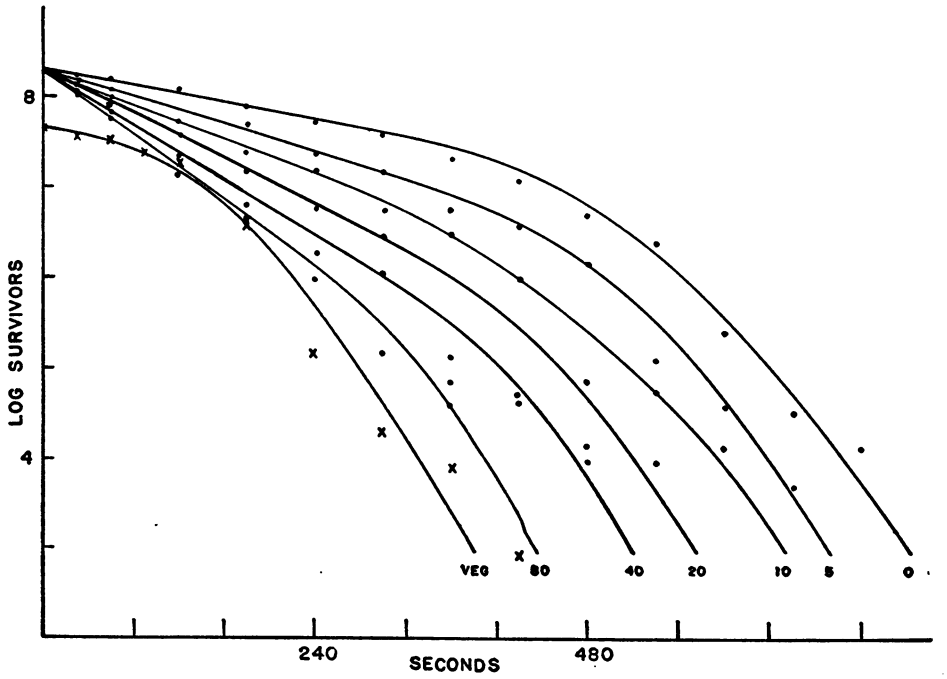

Figure 1. Survivor curves of *B. anthracis* spores germinated for the indicated time before exposure to ultraviolet light. Minutes of germination time are indicated by the figure under each curve. Data from a log phase vegetative culture (veg) are inserted for comparison.

The nature of such changes is under investigation and will be reported later. A recent series of papers by Hills (1949*a*,*b*, 1950) reported an analogous study on changes in thermal sensitivity of germinating spores of the same species; his

data suggest that a longer incubation time was required prior to loss of resistance to heat than we observed with ultraviolet, but this may be a result of the difficulty of making measurements at very short time intervals with the more cumbersome heat-killing procedure.

Because of the great decrease in sensitivity to ultraviolet during the germination period in the growth cycle we decided to compare the mutagenic effect of irradiation during the various stages of germination, not on the basis of dosage

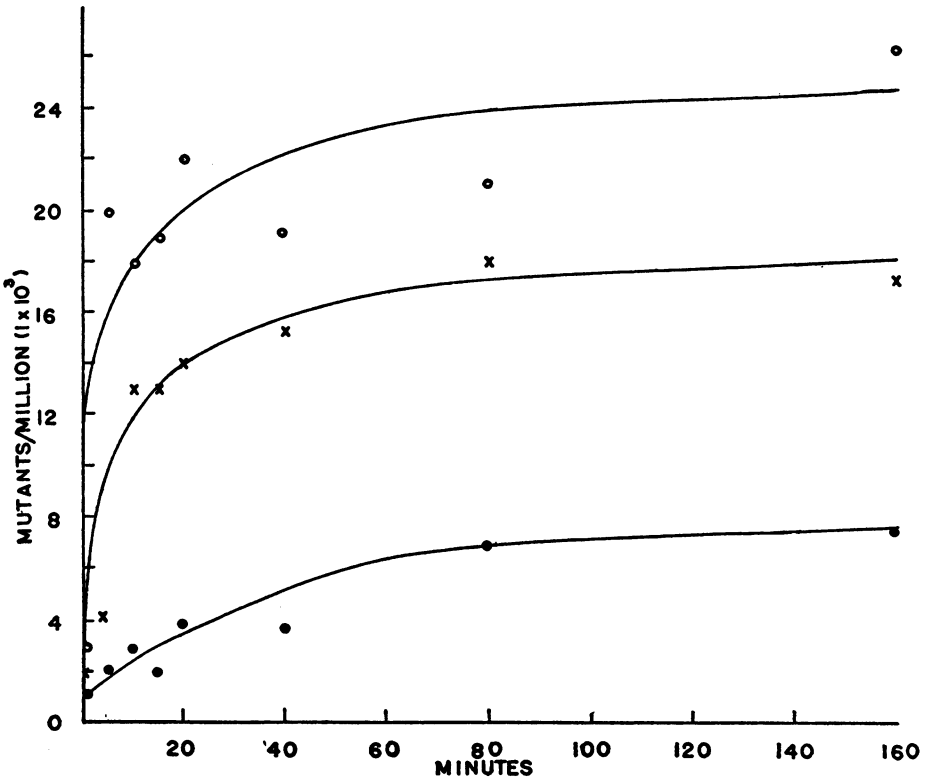

Figure 2. Incidence of mutants in spore populations subjected to 99.99 per cent killing as influenced by the time (minutes) allowed for germination prior to exposure to ultraviolet light. The three curves represent mutations to resistance to the following levels of streptomycin: dots, 2 μg per ml; crosses, 1 μg per ml; circles, 0.75 μg per ml.

but on the basis of lethal effect. In every experiment a closely spaced series of exposures to ultraviolet light was made, and those data were chosen for comparison where the lethality was of the order of 99.99 per cent. We have compared the number of mutants obtained from spores and vegetative cells and find that in all cases the mutation rate increased with increased killing; when 99.99 per cent of the cells were killed there was a greater proportion of mutants among the survivors than when 99.98 per cent were killed, and the latter group had a higher fraction of mutants than when 99.97 per cent killing occurred.

In figure 2 the incidence of mutants occurring with a 99.99 per cent kill is plotted against the time permitted for germination before the irradiation. Similar curves result when the 99.98 per cent or the 99.97 per cent lethality is used as a basis for comparing the induced mutation. The figure shows that the incidence of induced mutants, which are resistant to several levels of streptomycin, is low for the ungerminated spores but increases rapidly during the germination period. Spores permitted to germinate for only 5 minutes before exposure to ultraviolet light show a marked increase in the mutability, and this increase continues until a

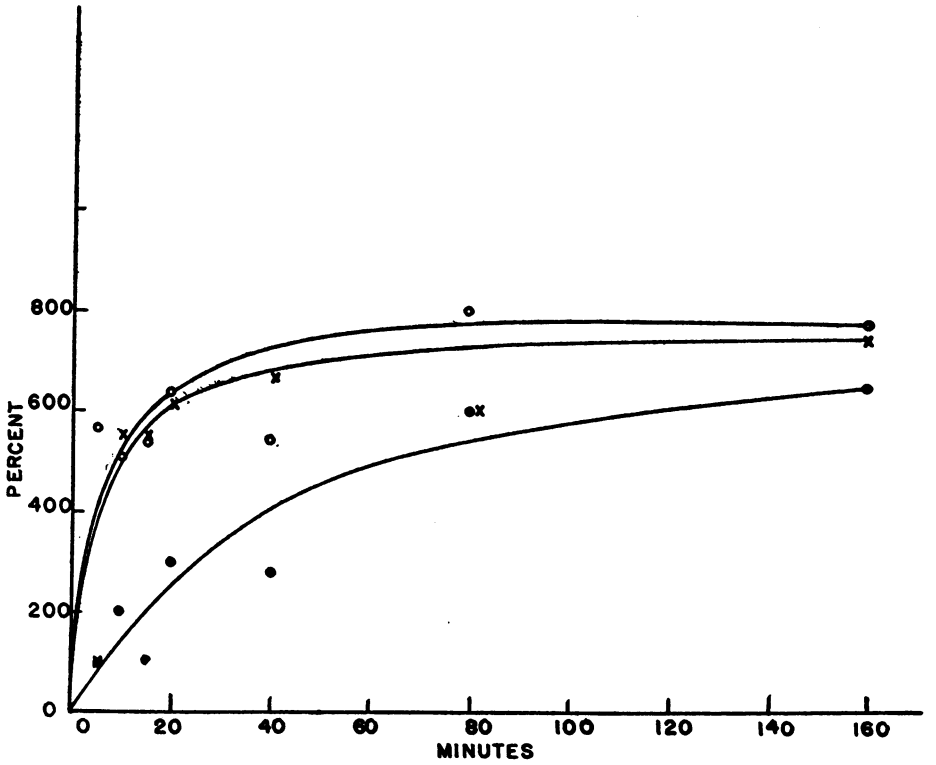

Figure 3. Percentage increase in mutations of *B. anthracis* spores as a function of the germination time (minutes) prior to treatment with ultraviolet.

leveling off becomes evident after 20 minutes in the germination medium. Further experiments showed that this relatively constant mutability persisted through the vegetative phase of growth and dropped only when spore formation occurred. Figure 3 shows the same data plotted on the basis of percentage of increase of mutations over that obtained with the ungerminated spores. This figure suggests that during the early germination period it is more difficult to induce mutations of very high resistance to streptomycin (resistant to 2 micrograms per ml) by a given exposure to ultraviolet irradiation.

The results plotted in figures 2 and 3 are for zero point mutations; that is, the organisms were plated to detect the mutants immediately after irradiation.

The irradiated survivors were also planted in fresh broth, and the proportion of mutants was determined on the resulting culture. On these data a correction for chain formation was applied and in general the results were less consistent; some delayed mutants appeared from the various stages of germination, but the shape of the curves was unchanged.

SUMMARY AND CONCLUSIONS

Our results are very similar to those of McElroy *et al.* (1947), who induced biochemical mutants in *Neurospora* with nitrogen mustard. With that organism the low mutation rate of old, dry conidia increased during germination, remained high through the vegetative state, and declined again as the conidia matured. These results differ from those obtained by Burkholder and Giles (1947), who reported about 3 per cent of biochemical mutants among the survivors of both vegetative cells and spores of *Bacillus subtilis* that had been subjected to a 99.9 per cent lethal exposure to ultraviolet.

Early in the germination period of sporogenous bacteria our organisms became more sensitive to ultraviolet irradiation. At the same time more mutations to streptomycin resistance were induced by a radiation dose of equivalent lethality. The mutability during the period of rearrangement of nuclear material in spore germination and in spore formation did not rise above that of the vegetative population. Because of the difficulty arising from chain formation our measurements on end point mutations are not so precise as those from germinating spores. They suggest, however, that in the culture developing from irradiated spores of *Bacillus anthracis*, delayed mutations occur just as they are found in vegetative cells.

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